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Lacrimal Gland Involvement in Blepharophimosis-Ptosis-Epicanthus Inversus Syndrome

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Purpose: To describe the involvement of the lacrimal gland (LG) in blepharophimosis-ptosis-epicanthus inversus syndrome (BPES).

Design: Observational, cross-sectional study.

Participants: Twenty-one patients with BPES (10 female, 11 male) aged on average 15 years (range, 2−39 years), from 3 Brazilian medical centers and 1 Portuguese medical center.

Methods: Patients had their ocular surface evaluated with slit-lamp biomicroscopy, and tear production quantified with the Schirmer test I. The LG volumes were measured on computed tomography (CT) scans in the BPES sample and in a group of age-matched subjects imaged for nonorbital diseases. Sixteen patients were screened for mutations in the *FOXL2* gene.

Main Outcome Measures: Lacrimal meniscus height, Schirmer test I, presence of superficial punctate keratopathy (SPK), LG volume, and molecular analysis of the *FOXL2* gene.

Results: Absence of LG was detected bilaterally in 9 patients (42.8%) and unilaterally in 2 patients (9.5%). When considering only patients with measurable LG, the median volume was 0.22 cm^3 in the right eye (range, 0.06−0.36 cm^3) and 0.24 cm^3 in the left eye (range, 0.08−0.34 cm^3). These values were significantly lower than those for the age-matched controls (median = 0.54 right eye and 0.53 left eye; $P < 0.05$). There was a significant association between deficiency of tear production and LG volume reduction and agenesis. Molecular analysis of the *FOXL2* gene revealed the presence of 8 distinct mutations, 4 of them novel ones. A significant reduction of LG size or agenesis was associated with mutations affecting protein size (due to underlying changes in the stop codon location) or the DNA-binding forkhead domain (Fisher exact test, $P = 0.021$). In 3 probands, the underlying genetic defect was not found.

Conclusions: This is the first study reporting LG volumes in BPES, describing a significant number of patients with LG agenesis. The association between alacrima and BPES is not incidental, and a thorough evaluation of tear production is recommended especially if ptosis surgery is planned. *Ophthalmology 2017;124:399-406 © 2016 by the American Academy of Ophthalmology*

Blepharophimosis-ptosis-epicanthus inversus syndrome (BPES) (Online Mendelian Inheritance in Man 110100) is an uncommon disorder first described in 1889 by Vignes.[1] Three decades later, Dimitry[2] defined its inheritance pattern as autosomal dominant, which was confirmed over subsequent years. Its molecular basis remained undetermined until 2001, when Crisponi et al[3] finally identified forkhead box protein L2 (*FOXL2*) (Online Mendelian Inheritance in Man *605597) on chromosome 3q23 as the causal gene.[3] This single-exon gene encodes a forkhead transcription factor expressed in the developing eyelid mesenchyme and fetal and adult ovaries.[4,5] Thus, depending on its expression, there are 2 types of BPES: type I with premature ovarian failure and type II with normal ovary function.[6,7] Severe ptosis with poor levator function, epicanthus inversus, and telecanthus are present in both types. We describe a significant spectrum of lacrimal gland (LG) changes in BPES. In addition, molecular analysis of

FOXL2 was performed in 16 patients in an attempt to obtain new insights into a possible genotype−phenotype correlation.

Methods

Patients

The research adhered to the tenets of the Declaration of Helsinki. Approval was obtained from the Institutional Review Boards and Ethics Committees, and all subjects gave written informed consent to participate in the study.

Twenty-one patients with a clinical diagnosis of BPES were recruited from 1 Portuguese and 3 Brazilian medical centers. Mean age was 15 years (range, 2−39 years). Schirmer test I (without anesthesia) and slit-lamp biomicroscopy assessment of the lacrimal meniscus height and corneal fluorescein staining were performed in all patients. A Schirmer's test score <10 mm after 5 minutes with

Table 1. Primers Used for Amplification and Sequencing of the Entire Coding Region of the *FOXL2* Gene

Primer Names	Primer Sequences (5'>3')	Size of the PCR Product (bp)	Annealing Temperature (°C)
FOXL2-1.1F	TTTGAGACTTGGCCGTAAGC	444	55
FOXL2-1.1R	TGATGAAGCACTCGTTGAGG		
FOXL2-1.2F	CGAAGTTCCCGTTCTACGAG	576	57
FOXL2-1.2R	CCAGGCCATTGTACGAGTTC		
FOXL2-1.3F	GTACACACGCGTGCAGAGC	407	57
FOXL2-1.3R	CGTCCCTGCATCCTCGCATC		

bp = base pair; PCR = polymerase chain reaction.

the eyes closed was considered as an indication of reduced aqueous tear production.[8,9] Two radiologists measured LG volumes on computed tomography (CT) scans using OsiriX software (developed by Pixmeo, SARL, Geneva; http://www.osirix-viewer.com) as the Digital Imaging and Communications in Medicine (DICOM) viewer. Briefly, as described by Bingham et al,[10] the LG area was measured from consecutive axial slices of orbital CT scans and added to obtain a final volume. The same protocol was applied to a group of 30 age-matched patients imaged for nonorbital pathology, which constituted the control group. The Wilcoxon signed-rank test was used to compare right and left LG volumes, and the Mann−Whitney test was used to compare LG volumes between groups. The Mann−Whitney U test was used to compare the Schirmer's test scores between eyes with nonmeasurable versus reduced LG. Finally, the Fisher exact test was used to analyze the association between the signs of keratopathy and LG size categorized as reduced and nonmeasurable. In all these tests, a P value ≤0.05 was considered significant.

DNA Amplification and Exon Sequencing

Sixteen patients were submitted to exon sequencing of the *FOXL2* gene. Genomic DNA was extracted from peripheral blood leukocytes using a QIAamp DNA Blood isolation kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For the analysis of the *FOXL2* gene, 3 pairs of primers were designed to cover the entire *FOXL2* exon and its splice site junctions (Table 1). The polymerase chain reaction−amplified DNA fragments were subjected to direct sequencing using the automatic capillary sequencing system ABI 3500X Genetic Analyzer (Applied Biosystems, Foster City, CA) and the Big Dye terminator v3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. The results were analyzed using the FinchTV version 1.4.0 software (Geospiza, Seattle, WA); the sequences obtained were compared with the reference from GenBank database (NM_023067), and the mutation nomenclature was used according to Human Genome Variation Society guidelines (http://www.hgvs.org/mutnomen). Pathogenic scores for missense mutations were calculated using the prediction tools SIFT,[11] MutPred,[12] and MutationTaster.[13]

Genotype−Lacrimal Gland Phenotype Correlation

To identify a possible genotype−phenotype correlation, 2 groups of mutations were considered: those affecting protein size or its functional domain and those not affecting the protein catalytic domain or its structure. Fisher exact test was used to determine the significance of the association between these 2 categories of mutations and LG abnormalities. A P value ≤0.05 was considered significant.

Results

Clinical and molecular data are shown in Table 2. Eight of the 21 patients were relatives belonging to 4 distinct families. Patients 1 and 2, 13 and 14, and 15 and 16 were progenitor and offspring, respectively, and patients 18 and 19 were twin brothers. Fifteen patients (71.4%) had already been submitted to some surgical procedure to correct their eyelid malformations. In 9 patients (42.8%), more than 1 procedure had been performed, including multiple revisions of silicone or autologous fascia slings for ptosis surgery. Twenty-two eyes (52.4%) from 12 patients showed clinical signs of reduced tear production manifested as decreased lacrimal meniscus height, low values on the Schirmer test, or superficial punctate keratopathy (SPK) of variable severity. Of these 22 eyes, 20 (90.9%) did not have a measurable LG ipsilaterally, and in the remaining 2 eyes, the LG size was markedly reduced.

A significant difference was found between the Schirmer's scores in absent versus reduced LG cases ($P = 0.0006$). Figure 1 shows the distribution of Schirmer's scores versus LG volumes. Eleven of the 14 eyes (78.6%) with absent LG showed Schirmer's values below the 10-mm cut off. In the group with reduced LG volumes, the Schirmer's scores were highly variable ranging from 3 to 35 mm. All the patients with normal LG presented normal Schirmer's values. A Schirmer's score less than 10 mm had a sensitivity of 68.4% to identify an LG volume of less than 0.20 cm³ (Fig 1). Finally, the Fisher exact test revealed that the presence of SPK was significantly associated with an absent LG ($P = 0.0007$).

Orbital CT scans disclosed LG agenesis in 11 patients, 9 (42.8%) bilaterally and 2 unilaterally. Figure 2 shows examples of the morphologic LG variants detected. With the exception of 3 patients (6 glands), all LG volumes were reduced and below the minimum value measured in the control group (Table 2). In regard to only patients with BPES with measurable LG (Fig 3), median LG volumes were 0.22 cm³ (range, 0.06−0.36 cm³) and 0.24 cm³ (range, 0.08−0.34 cm³) concerning the right and left sides, respectively, with no significant difference between sides ($P = 0.72$). In the control group, median LG volumes were 0.54 cm³ in the right orbits (mean 0.55 cm³; standard deviation, 0.19; range, 0.30−1.22) and 0.54 cm³ in the left orbits (mean, 0.53; standard deviation, 0.20; range, 0.32−1.29), values similar to those previously reported in the literature.[10] The Mann−Whitney test showed that the volumes of patients with BPES were significantly lower than in the control ($P < 0.05$) for both the right and left orbits.

Molecular Investigation

Molecular screening of the *FOXL2* gene showed pathogenic sequence variants in 13 (81.2%) of the 16 patients analyzed (Fig 4;

Table 2. Clinical, Imaging, and Molecular Analysis of Patients with Blepharophimosis-Ptosis-Epicanthus Inversus Syndrome

Patient	Age (yrs)	Gender	No. of Previous Surgeries	Laterality	Lacrimal Film Evaluation			Lacrimal Gland Imaging		FOXL2 Molecular Analysis		
					Lacrimal Meniscus	Schirmer I (mm)	Superficial Keratopathy	Glandular Volume (cm³)	Lacrimal Gland Size Rating*	cDNA	Protein	Type
1	14	F	1	R	+	18	N	0.24	Reduced	c.672_701dup30	p.A224-A234dup	In-frame duplication
				L	+	24	N	0.28	Reduced			
2	38	F	None	R	+	15	N	0.26	Reduced	c.672_701dup30	p.A224-A234dup	In-frame duplication
				L	+	17	N	0.29	Reduced			
3	11	F	3	R	↓	15	Diffuse	NM	Absent	Not detected	X	X
				L	↓	12	Diffuse	NM	Absent			
4	11	M	2	R	↓	2	Inferior	NM	Absent	Not detected	X	X
				L	+	12	N	0.24	Reduced			
5	13	M	1	R	+	32	N	0.29	Reduced	c.650C>G	p.S217C	Missense
				L	+	35	N	0.25	Reduced			
6	19	F	2	R	+	23	N	0.36	N	Not detected	X	X
				L	+	18	N	0.34	N			
7	10	F	1	R	+	> 35	N	0.32	N	c.672_701dup30	p.A224-A234dup	In-frame duplication
				L	+	> 35	N	0.32	N			
8	8	M	2	R	↓	7	Inferior	NM	Absent	c.292T>G	p.Y98G	Missense
				L	↓	11	Inferior	NM	Absent			
9	3	F	1	R	+	Unc	N	0.11	Reduced	c.172_176delTCGTA	p.S58RfsX36	In-frame deletion
				L	+	Unc	N	0.08	Reduced			
10	2	F	None	R	↓	Unc	Unc	NM	Absent	c.919_920insACCGCCGC	p.P307HFsX52	In-frame insertion
				L	↓	Unc	Unc	NM	Absent			
11	9	M	2	R	↓	3	Inferior	0.17	Reduced	c.370A>T	p.K124Stop	Nonsense
				L	↓	4	Inferior	0.12	Reduced			
12	2	M	None	R	↓	Unc	Unc	NM	Absent	c.768-769insG	p.P257AFs	In-frame insertion
				L	↓	Unc	Unc	NM	Absent			
13	39	M	None	R	↓	3	N	NM	Absent	c.313A>C	p.N105H	Missense
				L	↓	4	N	NM	Absent			
14	5	F	None	R	↓	Unc	Unc	NM	Absent	c.313A>C	p.N105H	Missense
				L	↓	Unc	Unc	NM	Absent			
15	36	M	2	R	+	22	N	0.12	Reduced	c.672_701dup30	p.A224-A234dup	In-frame duplication
				L	+	32	N	0.14	Reduced			
16	8	M	5	R	+	Unc	N	0.06	Reduced	c.672_701dup30	p.A224-A234dup	In-frame duplication
				L	+	Unc	N	0.16	Reduced			
17	7	F	1	R	+	18	N	0.10	Reduced	Not performed		
				L	+	6	Inferior	NM	Absent			
18	27	M	2	R	+	4	Inferior	NM	Absent	Not performed		
				L	↓	0	Inferior	NM	Absent			
19	27	M	2	R	↓	0	Inferior	NM	Absent	Not performed		
				L	↓	0	Inferior	NM	Absent			
20	31	F	None	R	NP	NP	N	0.32	N	Not performed		
				L	NP	NP	N	0.33	N			
21	2	F	1	R	↓	0	Inferior	NM	Absent	Not performed		
				L	↓	8	N	NM	Absent			

F = female; L = left eye; M = male; N = normal; NM = not measurable; NP = not performed; R = right eye; + = present; ↓ = visibly decreased; Unc = uncooperative.
*Comparative analysis with the control group: reduced when below and N when above the minimum value measured in the control group.
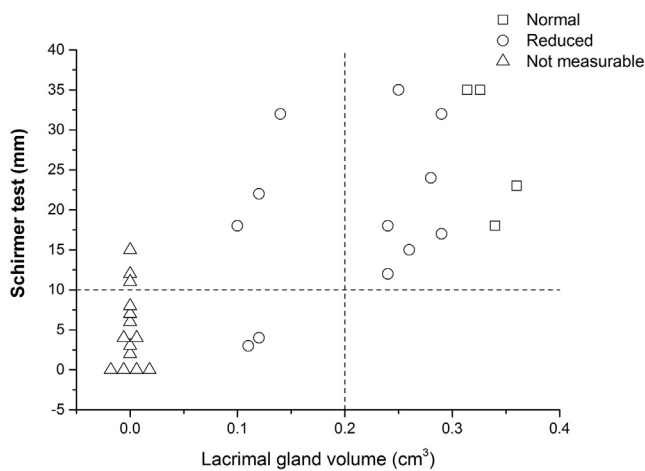
Figure 1. Distribution of Schirmer I test with lacrimal gland (LG) volumes.

Table 2). Eight distinct mutations were found, 4 of them previously unreported.

The most frequent mutation in this study was the in-frame duplication of 30 nucleotides, c.672_701dup30, resulting in a polyalanine expansion (p.A224-A234dup) that was detected in 5 patients (patients 1, 2, 7, 15, and 16) from 3 distinct families. Three missense mutations also were detected (c.650C>G, c.292T>G, c.313A>C), the last 2 in the DNA-binding forkhead domain of the gene.

Two in-frame insertions (c.919_920insACCGCCGC and c.768-769insG), a nonsense mutation (c.370A>T), and an in-frame deletion (c.172_176delTCGTA) were also found. All 4 interfered with the stop codon position; 3 of them led to a premature stop codon location (and abnormally small proteins), and the insertion from patient 12, c.768-769insG, deleted the wild-type stop codon of the gene, producing a hyper-long protein. Mutations c.172_176delTCGTA, c.370A>T, c.768-769insG, and c.313A>C from patients 9, 11, 12, and 13/14, respectively, were not previously described in BPES. The methodology used did not show any pathogenic mutations in 3 unrelated patients.

Genotype–Lacrimal Gland Phenotype Association

Six distinct mutations were associated with a more severe lacrimal phenotype, including absent or significantly reduced LG size and tear hyposecretion. These mutations comprised sequence variants inducing premature stop codons (c.172_176delTCGTA, c.919_920insACCGCCGC, and c.370A>T) or interfering with the wild-type one (c.768-769insG), and missense mutations located in the DNA-binding forkhead domain (c.292T>G, c.313A>C). The Fisher exact test showed a higher probability for these mutations to be associated with agenesis or underdevelopment of the gland ($P = 0.021$).

Lighter phenotypes, with LG volumes within or near control group values and normal tear evaluation, were detected in association with alanine expansions (c.672_701dup30) and a missense mutation just before the polyalanine tract of the *FOXL2* gene (c.650C>G).

In 3 unrelated patients, the gene sequence was normal. Two of these cases presented bilateral or unilateral LG agenesis (patients 3 and 4, respectively), and in the remaining one the gland was normal.

Discussion

The clinical presentation of BPES has been extensively described.[14] In addition to the classic signs of ptosis, epicanthus inversus and telecanthus, a variety of ophthalmic and nonophthalmic features[15–20] are variably associated with the syndrome. Deficiency of the tear production apparatus has been overlooked. To our knowledge, there are only 2 case reports of lacrimal hyposecretion. Athappilly and Braverman[21] described a 9-month-old female patient with BPES and bilateral absence of the LG. More recently, another 9-month-old infant with BPES was found to have absent tear production though normal-sized LGs were visible on ultrasound imaging.[22]

The present report is the first to show that the association between alacrima and BPES is not incidental. In our group of patients, 52.3% had no measurable LGs (42.8% bilaterally and 9.5% unilaterally), and 33% of the remaining cases showed reduced LG volume. Not surprisingly, there was a clear association between these radiologic findings and tear hyposecretion.

A genotype–phenotype correlation has not been proven as yet in BPES.[23] So far, more than 100 different mutations have been described involving *FOXL2*, and pathogenic variants can be found in approximately 88% of cases.[24] The presence of other loci causing the syndrome also has been proposed, although a clear association is still lacking.[17,25,26] We performed molecular screening of *FOXL2* in 16 patients to analyze specifically the LG phenotype. Thirteen patients (81%) showed pathogenic genetic changes, 4 of them already described by other authors[27-30] and the remaining 4 being novel mutations. The most frequent mutation was a 30-nucleotide duplication (c.672− 701dup30) causing an expansion of 10 alanine residues (p.A224−234dup) within the polyalanine tract (Fig 4). This mutation is responsible for a shift in the protein location from the nucleus, where it is normally located, to the cytoplasm, and induces a strong tendency to aggregation.[31] In our study, the same type of alanine duplication was found in 2 distinct families (1 of them Portuguese and the other Brazilian) and in 1 Brazilian sporadic case. This genetic defect is, in fact, recurrent and has been reported in distinct families with different geographic and ethnic backgrounds.[27]

We found 3 missense mutations, 2 of them, c.650C>G and c.292T>G, already known. The first[28] leads to a switch from the amino acid serine to cysteine, immediately upstream from the polyalanine domain. This substitution presumably acts as a loss-of-function mutation, producing hypomorphic or null alleles and haploinsufficiency.[29] The latter is a change from a tryptophan to a glycine within the forkhead domain of *FOXL2*, a more common location for missense mutations in BPES, and causes nuclear and cytoplasmic protein aggregation.[32] The third missense mutation (c.313A>C), found in 2 related patients, is a novel pathogenic variant and causes the substitution of an asparagine for a histidine in the forkhead domain of *FOXL2*.[24] The remaining 4 mutations included 2 insertions, c.919_920insACCGCCGC
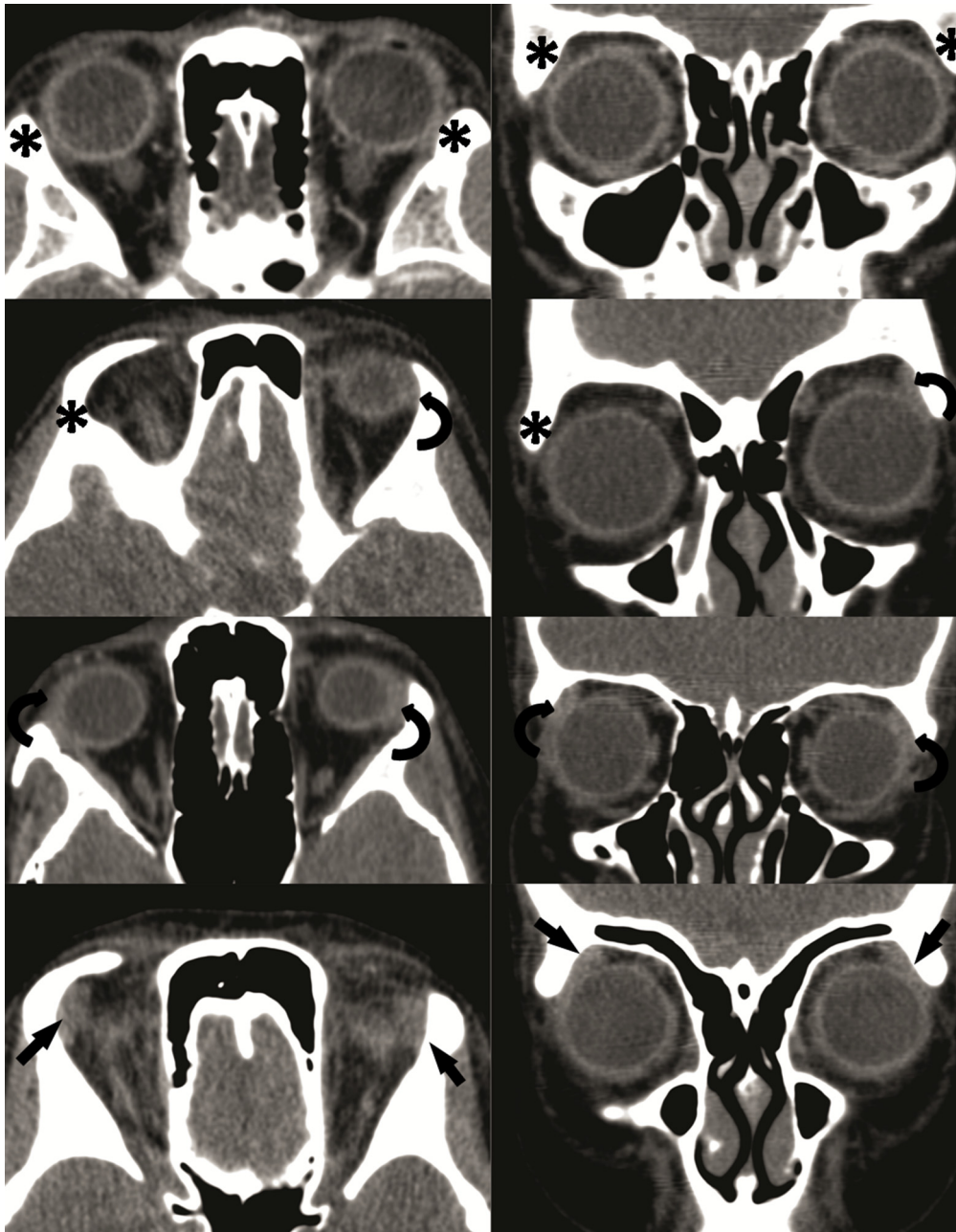
Figure 2. From top to bottom: axial (*left*) and coronal (*right*) computed tomography (CT) scans of 4 distinct patients. *Asterisks* demonstrate absence of the lacrimal gland (LG), *curved arrows* highlight reduced LG, and *straight arrows* indicate a normal-sized LG.

and c.768-769insG, a nonsense mutation, c.370A>T, and a deletion, c.172_176delTCGTA. Only the first insertion has been described.[30]

By taking into consideration only the LG phenotype and the mutation data, our data suggest that there is an association between the type of mutation and its location and LG abnormality. This finding needs to be corroborated by the evaluation of larger samples.

Overall, lower Schirmer scores in patients with BPES are highly suspicious for LG underdevelopment or even agenesia. The majority of patients with absent LG presented

subnormal Schirmer scores. Nonetheless, 3 eyes with not measurable LG displayed values above the 10-mm cut off. We believe that these higher scores might be the result of the presence of small amounts of lacrimal tissue indistinguishable in the CT scan. The LG is surrounded by numerous structures with different radiologic densities, which make the identification of a small quantity of lacrimal tissue difficult, even for trained radiologists.

In the group of eyes with reduced LG volumes, Schirmer scores were highly variable. One patient with very small LGs (patient 11) presented low scores; however, in the
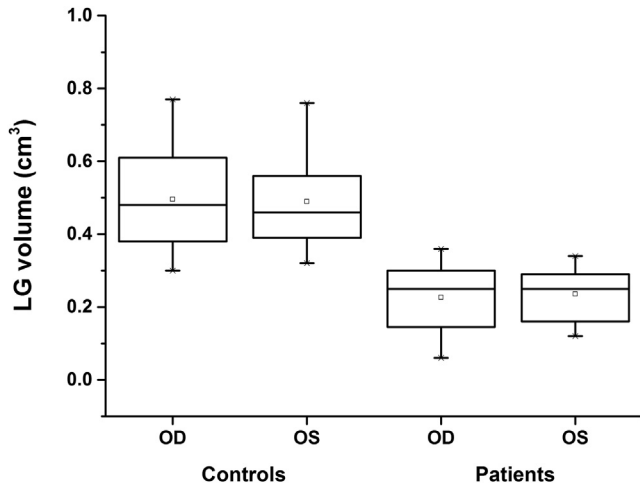
Figure 3. Box-plot diagrams showing the right eye and left eye lacrimal gland (LG) volume distribution in the control group (n = 30) and patients with blepharophimosis-ptosis-epicanthus inversus syndrome (BPES) (n = 21). The *central rectangle* represents the interquartile range, the *line* inside the *rectangle* represents the median, the *small square* indicates the mean, and the *whiskers* above and below the box show the minimum and maximum values. OD = right eye; OS = left eye.

remaining eyes there was not a clear relationship between LG size and Schirmer test. Some eyes with near-normal LG size displayed low Schirmer scores. When interpreting these results, one should keep in mind the unreliable nature and high variability of the Schirmer test.[33] Besides that, our data combined with a previous reported case of alacrima in a child with BPES and normal-sized LG[22] might indicate that *FOXL2* mutations have an impact not only in the size but also in the gland's function.

Three patients (numbers 6, 7, and 20) displayed normal LG volumes. Schirmer test was performed in 2 of these patients with normal scores. We could not find an association between normal-sized LG and the molecular results. One of the patients (number 6) did not have an identifiable mutation, patient 7 presented the alanine expansion c.672−701dup30, also associated with reduced size LG (Table 2), and in patient 20 the genetic screening was not performed.

The prevalence of LG underdevelopment in BPES remains unknown. In a previous study involving clinical, radiologic, and genetic analysis of 33 Indian patients with BPES, Chawla et al[17] used CT scans to study bone development and to compare orbital biometry with age-matched controls. No changes were detected in the LGs, which were reported to be apparently normal. We believe that a more accurate analysis of glandular volumes probably could show significant differences compared with the age-matched control group. However, it is interesting to notice that the rate of intragenic mutations in this group of patients with BPES was only 4% (1/25), which is below the usually reported rate of 72% in western countries.[30] As Chawla et al[17] suggest, this may have an ethnic reason, with the existence of other locus not yet described responsible for this distinct genetic background.

Considering our results, we must emphasize the importance of LG agenesis for the surgical management of BPES. There is a significant burden for the parents of affected children, who are usually extremely concerned about the
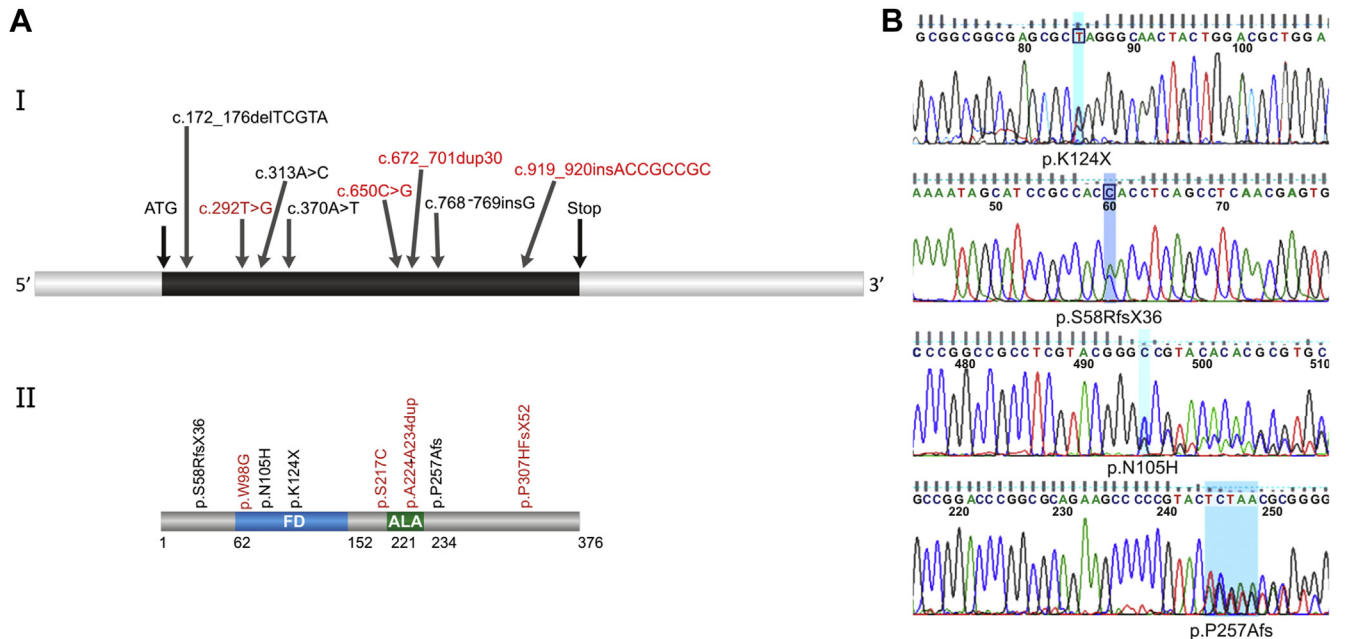

Figure 4. *FOXL2* mutations causing blepharophimosis-ptosis-epicanthus inversus syndrome (BPES). **A,** Organization of the human *FOXL2* gene (I) and its protein product (II). Mutations in black = mutations described in the current article; mutations in red = mutations previously described in the literature. Forkhead domain, poly-ALA. **B,** Representative chromatograms of novel *FOXL2* mutations. K124X (nonsense), S58RfsX36 (in-frame 5 amino acid deletion), N105H (missense), and P257Afs (in-frame 1 amino acid insertion). FD = forkhead domain.

abnormal appearance of the patients and exert a tremendous pressure for early cosmetic rehabilitation. Although there is no consensus about the best surgical strategy for the correction of the multiple palpebral fissure anomalies in BPES,[14] early ptosis surgery may be needed to alleviate the chin position and avoid visual deprivation.[34] Ptosis in BPES is generally severe, with poor to zero levator function, and most authors recommend bilateral frontalis slings. Supramaximal levator resection[35] and frontalis muscle flaps[36,37] have been attempted with variable results. For these reasons, a reduced tear production is a serious concern for the long-term effects of ptosis correction on the ocular surface, because it seems reasonable to accept that spontaneous blink amplitude might be reduced after all these different modalities of surgery.[38] In addition, a certain degree of nocturnal lagophthalmos is the rule after congenital ptosis surgery, increasing the odds of corneal complications. In our cohort, all patients with demonstrable LG absence had persistent SPK; however, we cannot ignore the influence of postsurgical lagophthalmos in this corneal staining.

In conclusions, tear film evaluation often is neglected in children with BPES mainly because of the low position of the upper lid and the lack of cooperation. Nevertheless, our findings support the need for a careful preoperative evaluation of the lacrimal production status in all patients with BPES. If there is any sign of SPK or a history of tear absence when crying, orbital imaging should be considered to confirm LG underdevelopment.

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Footnotes and Financial Disclosures

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Abbreviations and Acronyms:

BPES = blepharophimosis-ptosis-epicanthus inversus syndrome; **CT** = computed tomography; **FOXL2** = forkhead box protein L2; **LG** = lacrimal gland; **SPK** = superficial punctate keratopathy.

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